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MEDIAN LETHAL DOSES ASSOCIATED WITH INTRAVENOUS EXPOSURE TO THE OPTICALLY PURE ENANTIOMERS OF VX IN GUINEA PIGS

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Stereoisomers are molecules with the same sequence of bonded atoms but a different orientation of these atoms in space, and enantiomers are non-superimposable stereoisomers. Enantiomers have the same chemical and physical properties except for their ability to rotate linearly polarized light in opposite directions. Nearly all previous research with chemical warfare nerve agents has been conducted using a mixture containing equal amounts of each stereoisomer or enantiomer, but it has been hypothesized that the stereoisomers themselves may yield different toxic responses. Thus, we estimated the 24 h median lethal doses for intravenous exposure of adult, male guinea pigs to the individual VX enantiomers, and we compared those potencies to that for a racemic mixture. The P(+)-isomer of VX was 60× less toxic than the P(–)-isomer and 50× less toxic than the racemic mixture. However, the P(+)-isomer would still be considered highly toxic based on the U.S. Environmental Protection Agency's Acute Toxicity Categories for Pesticide Products.

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PREFACE

The work described in this report was authorized under project no. CB3281. The work was started in January 2016 and completed in July 2016, as recorded in ECBC notebook 15-0101 (U.S. Army Edgewood Chemical Biological Center; Aberdeen Proving Ground, MD).

In conducting the research described in this report, the investigators adhered to the *Guide for the Care and Use of Laboratory Animals* (National Academies Press: Washington, DC, 2016). These investigations were also performed in accordance with the requirements of AR 70-18, *The Use of Animals in DoD Programs* (Laboratory Animals, Procurement, Transportation, Use, Care and Public Affairs), and the Institutional Animal Care and Use Committee (IACUC), which oversees the use of laboratory animals by reviewing for approval all ECBC research protocols requiring laboratory animals. This project, assigned IACUC protocol no. 16-472, was approved in December 2015.

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This report has been approved for public release.

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1. INTRODUCTION

The center phosphorus atom of VX (*O*-ethyl *S*-[2-(diisopropylamino)ethyl] methylphosphonothioate) rotates linearly polarized light both clockwise [P(+)-isomer] and anticlockwise [P(–)-isomer], so that its synthesis results in equal amounts of two enantiomers with the same chemical and physical properties. The toxicological properties of the two enantiomers, however, are anticipated to be different because they exert their effects in a chiral, biologic environment. As reviewed by Benschop and De Jong (1988, 2001), the biomolecular reaction rate constant for the inhibition of bovine acetylcholinesterase by the P(+)-isomer of VX is 2 orders of magnitude lower than that by the P(–)-isomer. The median lethal dose (LD_{50}) for mice intravenously exposed to the P(+)-isomer of VX is also 1 order of magnitude higher than that for the P(–)-isomer, and the LD_{50} for the P(–)-isomer is approximately half that of the racemic mixture (Benschop and De Jong, 1988, 2001). To our knowledge, these are the only studies involving administration of the optically pure enantiomers of VX to an animal model. Mice, however, are not ideal for studying the toxicity associated with nerve agent exposure because they have relatively high levels of carboxylesterase activity as compared with humans (Maxwell et al., 1987). Guinea pigs have lower levels of carboxylesterase activity than mice (Maxwell et al., 1987); thus, we estimated the 24 h LD_{50} values for intravenous exposure of guinea pigs to optically pure enantiomers of VX, and we compared the potencies with those for the racemic mixture.

2. METHODS

2.1 Animals

Adult, male guinea pigs (body weight, 350–400 g) with surgically implanted jugular vein catheters were purchased from Charles River Laboratories International, Inc. (Kingston, NY). Guinea pigs were single-housed in temperature- and humidity-controlled rooms (21 ± 1 °C and 50 ± 20%, respectively) with the lights on from 0600 to 1800. Food and water were provided ad libitum, and the animals had access to enrichment items. Guinea pigs weighed 456 ± 37 g (mean ± standard deviation) at the time of the VX exposures, which occurred no less than 5 days after their arrival.

2.2 Agent

Optically pure enantiomers were separated from the racemic VX mixture by members of the Agent Chemistry Branch (U.S. Army Edgewood Chemical Biological Center; Aberdeen Proving Ground, MD) using procedures described by Bae and Winemiller (2016). An aliquot of neat (undiluted) agent was transferred from the Agent Chemistry Branch to the Operational Toxicology Branch on the morning of each exposure (approximately 30 min before

the first guinea pig was exposed to the agent under investigation). The purity of the agent in each aliquot was verified by nuclear magnetic resonance spectroscopy no more than 24 h before it was transferred between branches.

2.3 Exposures

Guinea pigs (n = 4–10 animals per group) were intravenously exposed via their catheters to one of the optically pure enantiomers or the racemic mixture of VX. The solvent used was saline, and the injection volume was 0.5 mL/kg. The guinea pigs were exposed in a staircase fashion to no more than six doses of each agent, ranging from 175 to 280 µg/kg for the P(+)-isomer, 3.0 to 5.0 µg/kg for the P(–)-isomer, and 3.7 to 7.0 µg/kg for the racemic mixture of VX. Exposures were discontinued once there was a stable probit solution permitting an interpolative estimate of the 24 h LD_{50} value for each agent. Toxic signs (convulsions, lacrimation, muscle fasciculations, salivation, tremors, etc.) were continuously monitored for the first 2 h post-exposure and then every 30–60 min until the end of the business day. At 24 h post-exposure, guinea pigs were intravenously administered a barbiturate euthanasia solution (390 mg/mL of sodium pentobarbital). Once respiration ceased and a heartbeat could no longer be felt upon palpation, biosamples (blood, brain, heart, liver, lung, kidneys, and urine) were collected. Biosamples were also collected from guinea pigs that died during the business day. Blood was stored at –20 °C, whereas the other biosamples were stored at –80 °C until they could be analyzed by the Analytical Toxicology Branch.

2.4 Data Analyses

Toxic signs for each of the optically pure enantiomers and the racemic mixture of VX were categorized as moderate, severe, or lethal. Toxic signs were considered lethal if the animal died before the 24 h time point. Collapse, convulsions, gasping, and prostration were considered to be severe toxic signs, and any other toxic signs were considered moderate. A median effective dose (ED_{50}) was estimated for each agent and category of toxic signs by applying ordinal regression analyses (Agresti, 1990) and maximum likelihood estimations (Fox, 1997). Survival analyses were conducted in accordance with Kaplan and Meier's methods (1958), and survival curves were compared using a log-rank test. Probit dose–response models (Finney, 1971) were fitted to the lethality data, and 24 h LD_{50} values and 95% confidence limits (CLs) were estimated for each agent. Statistical analysis routines contained within Minitab 17 (Minitab, Inc.; State College, PA) and SigmaPlot 13.0 (Systat Software, Inc.; San Jose, CA) systems were used for the data analyses.

3. RESULTS

Toxic signs were observed in all but 12 of the guinea pigs intravenously exposed to VX; of these, 4 animals were exposed to the P(–)-isomer and 8 animals were exposed to the racemic mixture. Figure 1 shows the onset of toxic signs (mean ± standard deviation) for guinea pigs exposed to the highest dose of each agent; moderate signs were observed within the first 10 min post-exposure. Muscle fasciculations were typically the first toxic sign to be observed, which were followed by tremors and body jerks. Salivation, lacrimation, and tearing (a milky

white eye secretion) were among the other moderate signs that were observed. Severe signs were observed between 30 and 60 min post-exposure.

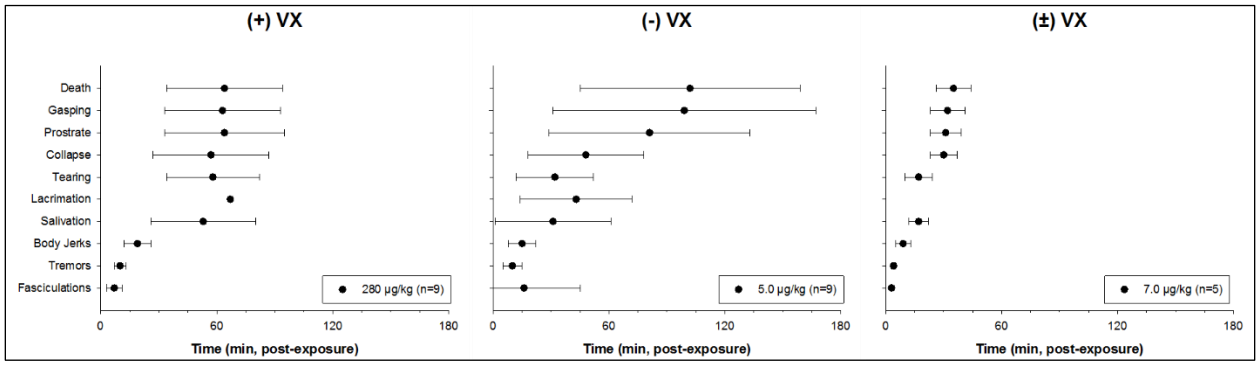

Figure 1. Onset of toxic signs for guinea pigs intravenously exposed to the highest dose of each optically pure enantiomer or the racemic mixture of VX.

Table 1 shows the ED_{50} values for moderate, severe, and lethal signs of toxicity for guinea pigs intravenously exposed to one of the optically pure enantiomers or the racemic mixture of VX. Because toxic signs were observed in all of the guinea pigs exposed to the P(+)-isomer of VX, an ED_{50} value could not be determined for moderate signs. Regardless, there was a statistically significant difference ($p < 0.001$) between the three agents in terms of their ED_{50} values. The lowest ED_{50} values were estimated for the P(–)-isomer of VX, whereas the highest ED_{50} values were estimated for the P(+)-isomer.

Table 1. Median Effective Doses for Guinea Pigs Intravenously Exposed to Optically Pure Enantiomers or Racemic Mixture of VX

Agent	Value	Slope ± SE	Toxic Sign Category		
			Moderate (µg/kg)	Severe (µg/kg)	Lethal (µg/kg)
(+)-VX	ED_{50} 95% CL	11.4 ± 3.2	N/A	243.4 224.4–264.0	268.4 243.1–296.2
(–)-VX	ED_{50} 95% CL	11.3 ± 2.8	2.8 2.4–3.4	3.6 3.2–4.0	4.3 3.9–4.8
(±)-VX	ED_{50} 95% CL	14.7 ± 3.2	4.0 3.7–4.4	4.7 4.3–5.1	5.4 4.9–5.9

N/A, not applicable; SE, standard error.

Figure 2 shows the survival curves for guinea pigs intravenously exposed to one of the optically pure enantiomers or the racemic mixture of VX. Time of death was arbitrarily set at 1200 min for those guinea pigs that died overnight. Kaplan–Meier analysis revealed a statistically significant difference ($\chi^2 = 59.681$; $p < 0.001$) between the survival curves for the P(+)-isomer; the 280 µg/kg group was significantly different ($p < 0.001$) from the other four groups, with a survival time of 64.6 ± 30.4 min. A significant difference ($\chi^2 = 25.137$; $p < 0.001$)

between survival curves was found for the P(–)-isomer; the 5.0 µg/kg group was significantly different from the 3.0 µg/kg ($p < 0.001$) and 4.2 µg/kg ($p < 0.01$) groups, with a survival time of 102.1 ± 57.4 min (excluding one guinea pig that survived to the 24 h time point). A significant difference ($\chi^2 = 32.868$; $p < 0.001$) between survival curves was also found for the racemic mixture; the 7.0 µg/kg group was significantly different from the 3.7 µg/kg ($p < 0.05$) and 5.0 µg/kg ($p < 0.01$) groups, with a survival time of 35.8 ± 8.9 min.

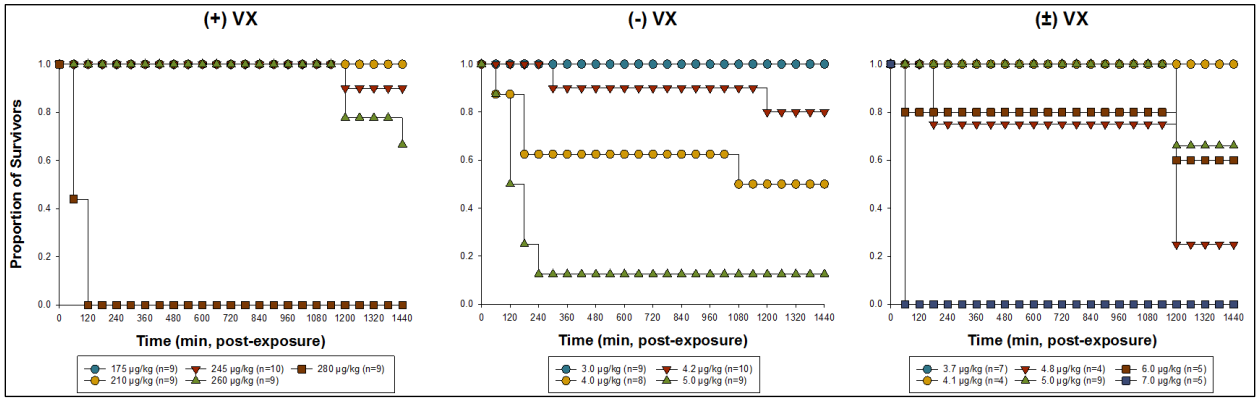

Figure 2. Survival curves for guinea pigs intravenously exposed to one of the optically pure enantiomers or the racemic mixture of VX.

Figure 3 shows the dose–response (lethality) curves that were generated during the probit analyses for guinea pigs intravenously exposed to one of the optically pure enantiomers or the racemic mixture of VX. In the figure, the dose–response curve for the P(+)-isomer of VX is shifted to the right as compared with the curves for the P(–)-isomer and the racemic mixture. The 24 h LD_{50} value for the P(+)-isomer was estimated to be 261.3 µg/kg, with a 95% CL of 254.4 to 271.3 µg/kg and a probit slope of 58.1 ± 17.6 (slope ± standard error). The 24 h LD_{50} value for the P(–)-isomer was estimated to be 4.4 µg/kg, with a 95% CL of 4.0 to 4.8 µg/kg and a probit slope of 16.7 ± 6.0. The 24 h LD_{50} value for the racemic mixture was estimated to be 5.4 µg/kg, with a 95% CL of 4.9 to 6.4 µg/kg and a probit slope of 11.7 ± 3.7.

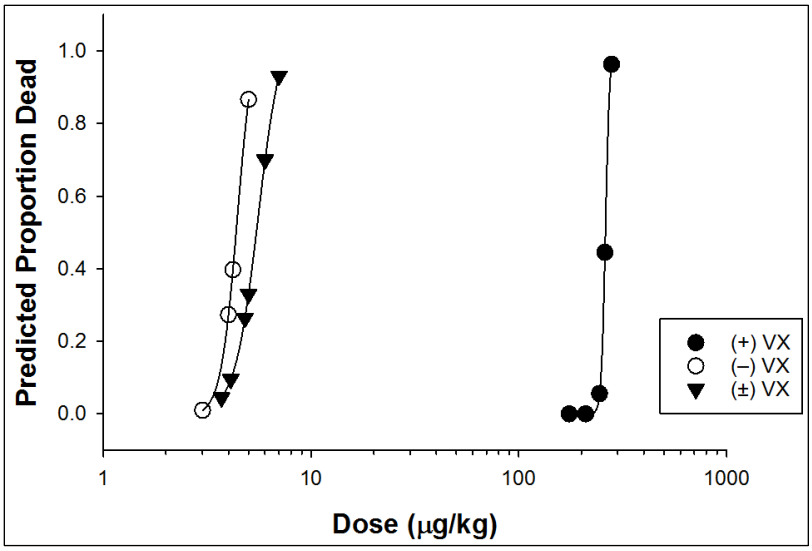

Figure 3. Probit dose–response curves for guinea pigs intravenously exposed to one of the optically pure enantiomers or the racemic mixture of VX.

4. DISCUSSION

Nearly all previous research with VX has been conducted with a racemic mixture of VX enantiomers; however, it has been hypothesized that the enantiomers themselves may have different toxic responses, given the binding differences with acetylcholinesterase. The reactivity of bovine (Benschop and De Jong, 1988, 2001), as well as human (Ordentlich et al., 2004) and swine (Reiter et al., 2008), acetylcholinesterase toward the P(+)-isomer of VX is 2 orders of magnitude lower than that toward the P(–)-isomer. However, van der Schans and colleagues (2003) found no stereospecificity by the two enantiomers of VX with respect to the rates of acetylcholinesterase inhibition from the erythrocytes of guinea pigs. Moreover, they found no stereospecificity in the sequestration of the two VX enantiomers following the intravenous administration of the racemic mixture to both guinea pigs and marmosets. Thus, additional in vivo research is needed to determine whether there is a difference in toxicity between the enantiomers.

In this study, we intravenously exposed adult, male guinea pigs to each of the optically pure enantiomers of VX, as well as a racemic mixture, and collected lethality data. The dose-response curve for the P(+)-isomer of VX was shifted to the right compared to the curves for the P(–)-isomer and the racemic mixture, which confirms Benschop and De Jong's (1988, 2001) findings in mice that this isomer is the least potent. The 24 h LD_{50} value for the P(+)-isomer of VX was estimated to be 261.3 µg/kg, which is 1–2 orders of magnitude higher than the values estimated for the P(–)-isomer and the racemic mixture (4.4 and 5.4 µg/kg, respectively). The magnitude of the difference between the two enantiomers was higher than that found in the only other in vivo studies to evaluate their toxicity (Benschop and De Jong, 1988, 2001); however, guinea pigs are a better animal model than mice for evaluating the toxicity of anticholinesterases due to their lower levels of carboxylesterase (Maxwell et al., 1987).

In Benschop and De Jong's mouse studies with the enantiomers of sarin, soman, tabun, and VX (1988, 2001), the LD_{50} values for the P(–)-isomers were approximately half of those for the racemic mixtures. Although we found a significant difference between the LD_{50} values for the P(–)-isomer and the racemic mixture of VX, the difference was not as great as expected. We expected the LD_{50} value for the racemic mixture to be higher, considering that van der Schans et al. (2003) had reported a value of 28 µg/kg for intravenous VX exposure in guinea pigs. However, those guinea pigs were hairless, and they were anesthetized with ketamine hydrochloride, which has been proposed as a treatment for nerve agent-induced seizures (reviewed by Dorandeu et al., 2013) when administered prior to the exposure. In addition, LD_{50} values between 8 and 11 µg/kg have been reported for subcutaneous exposure to VX in guinea pigs (Atchison et al., 2004; Shih and McDonough, 2000; Wetherell et al., 2002). Nonetheless, the rank order of toxicity for VX was consistent with that identified in previous studies (Benschop and De Jong, 1988, 2001): P(–)-isomer > racemic mixture > P(+)-isomer.

In summary, the P(+)-isomer of VX was 60× less toxic than the P(–)-isomer and 50× less toxic than the racemic mixture. It would still be considered highly toxic, based on the U.S. Environmental Protection Agency's Acute Toxicity Categories for Pesticide Products; therefore, its toxicity should not be discounted in future investigations.

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ACRONYMS AND ABBREVIATIONS

CL	confidence limit
ED_{50}	median effective dose
LD_{50}	median lethal dose
N/A	not applicable
SE	standard error
VX	*O*-ethyl *S*-[2-(diisopropylamino)ethyl] methylphosphonothioate
(+)-VX	P(+)-isomer of VX
(–)-VX	P(–)-isomer of VX
(±)-VX	racemic mixture of VX

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